

AMENDMENTS TO THE SPECIFICATION

Please amend the specification as indicated hereafter. It is believed that the following amendments and additions add no new matter to the present application.

In the Abstract:

Please replace the pending abstract with the newly-submitted abstract attached herewith on a separate sheet.

In the Specification

Please amend the paragraph starting on p. 12, line 14 as follows:

Accordingly, in a second aspect the invention relates to a type I repeated thyroglobulin domain inhibitor peptide with activity towards aspartic proteases, said peptide having the amino acid sequence extending from amino acid position 68-199 of equistatin of Fig. 1 (SEQ ID NO:2) or a modified type I repeated thyroglobulin aspartic protease inhibitor peptide wherein said modified peptide comprises a peptide having substantial amino acid identity to amino acid position 68-199 of equistatin; truncations of amino acid position 68-199 of equistatin; or truncations of the peptide having substantial amino acid identity to amino acid position 68-199 of equistatin, wherein said modified peptide is functionally equivalent to said amino acid position 68-199 of equistatin with aspartic protease inhibitor activity.

Please amend the paragraph starting on p. 18, line 18-20 as follows:

primers for the amplification of equistatic cDNA (SEQ ID NO:5 and SEQ ID NO:6, respectively)

EI-deg1: CR (A,C,G,T) AC (A,C,G,T) AA (A,G) TG (T,C) CA (A,G) CA (A,G)

EI-deg2: ATT (A,G) AC (A,G,C,T) TG (A,C,G,T) GG (A,C,G,T) CG (T,C) T-T (A,G) AA

Please amend the paragraph starting on p. 18, line 21 as follows:

As can be seen from SEQ ID NO:1 and SEQ ID NO:2 (Figures 1 and 2, respectively) the mature protein component of equistatin is composed of 3 domains that appear to have resulted from the duplication of genetic material. On the basis of preliminary cDNA sequence analysis, several structural isoforms of equistatin may occur in the *Actinia equina*. The 3 domains comprise a 22 kD

polypeptide. Each domain comprises about 65-68 amino acids, with 3 presumed disulphide bonds. Based on the sequences of the domains, it is apparent that the protein is a member of the conserved type I repeated thyroglobulin domain comprising repeating type I domains. Specifically the domain sequences show high conservation of the amino acid sequence (SEQ ID NO:7): Cys-(Xxx)₁₈₋₂₉-Pro-Xxx-Cys-(Xxx)₃-Gly-(Xxx)₅-Gln-Cys-(Xxx)₆-Cys-Thr-Cys-Val-(Xxx)₃-Gly-(Xxx)₁₀₋₁₅-Cys. The three domain inhibitors purified from *Actinia equina* was proteolytically cleaved into two major peptides and separated by reverse phase HPLC. Determination of the N-termini of both fragments allowed them to be located in the sequence. One peptide designated eqd-1 consisted of the first domain running from residue 1-67, whereas the second peptide designated eqd-2,3 contained domains 2 and 3 with residues 68-199. The intact equistatin molecule could be inhibited by only 1 papain and 1 cathepsin D molecule. Inhibition assays with Eqd-1 and Eqd-2,3 determined that Eqd-1 could only be inhibited by papain and Eqd-2,3 only with Cathepsin D. The inhibition constants for the separated domains were similar to the intact equistatin molecule. This demonstrated that even though these domains appear to be structurally conserved, that the specificities for proteases has diverged to completely different classes of proteases. It is not possible with the present evidence to know which residues determine this difference in specificities.

Please amend the paragraph starting on p. 19, line 29 and ending on p. 20, line 27 as follows:

An "effectively homologous variant" of a molecule such as the equistatin molecule is meant to refer to a molecule substantially similar in sequence and function to either the entire molecule or to a fragment thereof. For purposes of this invention, these molecules are identified when they contain the type I repeated thyroglobulin domain. Generally, the effectively homologous sequences should retain high conservation at the naturally occurring positions of the conserved sequence at SEQ ID NO:7: Cys-(Xxx)₁₈₋₂₉-Pro-Xxx-Cys-(Xxx)₃-Gly-(Xxx)₅-Gln-Cys-(Xxx)₆-Cys-Thr-Cys-Val-(Xxx)₃-Gly-(Xxx)₁₀₋₁₅-Cys. The two cysteines on either end of the conserved sequence are conserved, but they do not have conserved positions. They are likely to form structurally important disulphide bridges with any one of the other cysteines, however, for which reason they are included. For purposes of this invention, the structure of one amino acid sequence is effectively homologous to a second amino acid sequence if at least 70 percent, preferably at least 80 %, and most preferably

at least 90 % of the active portions of the amino acid sequence are identical or equivalent. General categories of potentially equivalent amino acids are set forth below, wherein, amino acids within a group may be substituted for other amino acids in that group: (1) glutamic acid and aspartic acid; (2) lysine, arginine and histidine; (3) alanin, valine, leucine and isoleucine; (4) asparagine and glutamine; (5) threonine and serine; (6) phenylalanine, tyrosine and tryptophan; and (7) glycine and alanin. More importantly and critical to the definition, the function of a second amino acid sequence is effectively homologous to another amino acid sequence if the second amino acid conforms to a tertiary structure having the capacity to decrease or eliminate the catalytic activity of a digestive cysteine and/or aspartic protease.

Please amend the paragraph starting on p. 39, line 10 as follows:

The kinetics of binding of equistatin to cathepsin D was performing using a synthetic substrate which contains a chromophore, such as a nitrophenylalanine residue, in the P1' position. The assay sensitivity, afforded by H-Pro-Thr-Glu-Phe*Nph-Arg-Leu-OH (SEQ ID NO:8) as substrate, allowed us to use 6.4 nM concentration of the enzyme as a minimal concentration in the test. The obtained equilibrium dissociation constant for the interaction between cathepsin D and equistatin ($K_i = 0.3$ nM) indicates that equistatin is a remarkably good inhibitor of aspartic proteinase, cathepsin D. For the papain active fragment, eq d-1, the K_i approx. 1mM was determined. This value is several orders of magnitude higher than is the K_i value for the intact equistatin, indicating that the inhibitory active site of the equistatin must be located on other domains. The eq d-2,3 indeed exhibited practically the same inhibition characteristics as the whole equistatin ($K_i > 0.6$ nM). Additionally, the formation of a tight complex between cathepsin D and equistatin was also visualized by a native PAGE (Fig. 10B).

Please amend the paragraph starting on p. 43, line 9 as follows:

Two primers were used to amplify the mature protein of equistatin and to clone it as an NcoI-NotI fragment behind the g3 signal peptide present in the E.coli expression vector pB3. Primer 1 was PDEI-1: CGC GCC ATG GCG AGT CTA ACC AAA TGC CAA (SEQ ID NO:9) and primer 2 was PDEI-2: GGG TGC GGC CGC GCA TGT GGG GCG TTT AAA (SEQ ID NO:

10). Correct inserts were sequenced to check for sequence errors and one clone was selected for obtaining recombinant protein.

Please amend the paragraph starting on p. 60, line 14 as follows:

PCR-primers used for PCR amplication (SEQ ID NO:11 – SEQ ID NO:16).

Please amend the paragraph starting on p. 63, line 7 as follows:

The 6 amino acid residues Gly-Tyr-Cys-Trp-Cys-Val (SEQ ID NO:17) which are strongly conserved among type I repeated thyroglobulin cysteine and aspartic protease inhibitors, whether from human, salmon or sea anemone sources, may be used to isolate homologous sequences with improved specificities. Degenerate PCR primers may be designed based on these sequences to amplify genomic or cDNA fragments which can be used as probes to isolate the entire coding sequence from for example cDNA libraries or by 5'RACE experiments from purified mRNA. Any organism including insects and plants may be used as new sources of type I repeated thyroglobulin domains. Collections of genes may be used in gene shuffling experiments to isolate new specificities.